ImageCalc: A microsoft® windows(TM) application for quantitative image analysis and comparison

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ImageCalc: A Microsoft® Windows™ Application for Quantitative Image Analysis and Comparison

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ABSTRACT

In this report we describe a Microsoft® Windows™-based computer program (ImageCalc) for the analysis of gels and autoradiograms and for computation of stand-alone images and images that are related to each other (i.e., images with the same image parameters). The program is able to subtract, add, multiply and divide constant values or full images from another image. It measures the intensity of (part of) an image in two ways: (i) *by calculation of the total intensity and the average intensity/pixel and* (ii) *by line scanning. The program allows importation of all images that are stored in an 8-bit uncompressed format and saves them in a Microsoft Windows bitmap format. Densitometric analysis of gels and autoradiograms, digitized using an ordinary optical scanner, is illustrated for glycosaminoglycans separated by electrophoresis on agarose gels and stained with Azure A silver, and for [^{32}P]phosphatidic acid, separated by thin-layer chromatography. Another application of the program is the analysis of multiple related images that are resolved in time or for which different emission or excitation wavelengths are used (fluorescence microscopy). As an example, the change of cytosolic [Ca^{2+}] is demonstrated in cultured human skeletal muscle cells after stimulation with acetylcholine.*

INTRODUCTION

Digitalization of images is a powerful tool for analyzing two-dimensional objects. In biochemistry, digitalization and analysis of gels and autoradiograms is a useful application. For this purpose, commercial programs are available that usually use very expensive scanning devices (e.g., laser scanners) in combination with sophisticated computer systems and/or software. However, laser scanners cannot be used for quantitative analysis of polychromatic or weakly stained silver images (4). Digitalization using ordinary scanners applying white light and detection by charge-coupled device (CCD) chip are more convenient in this respect. To our knowledge, no public domain programs for the analysis of digitalized images exist for Microsoft® Windows™-based computers, although some are available for Macintosh® computers (e.g., NIH Image) (5). We therefore developed a Microsoft Windows-based computer program for the densitometric analysis of gels and autoradiograms. The program is also useful for the analysis of standalone and related (microscopic) images.

METHODOLOGY

ImageCalc was written in Borland Pascal as an application for Microsoft Windows (Microsoft Corporation, Redmond, WA, USA). Windows applications allow the simultaneous visualization and manipulation of several images and facilitate the analysis of related images. All image types, which are stored in an uncompressed 8-bit format (e.g., TIFF files) can be imported. The applicability of the program is very general since almost every digitalization device supports such a format. The program can be used to perform arithmetic calculations on single images and on two related images. Specific areas of an image (e.g., silver-stained bands or parts of a cell) can be selected and analyzed. Analysis of the same area in different related images is possible and is especially useful for the analysis of (microscopic) images that are resolved in time or that differ in the emission/excitation wavelengths used.

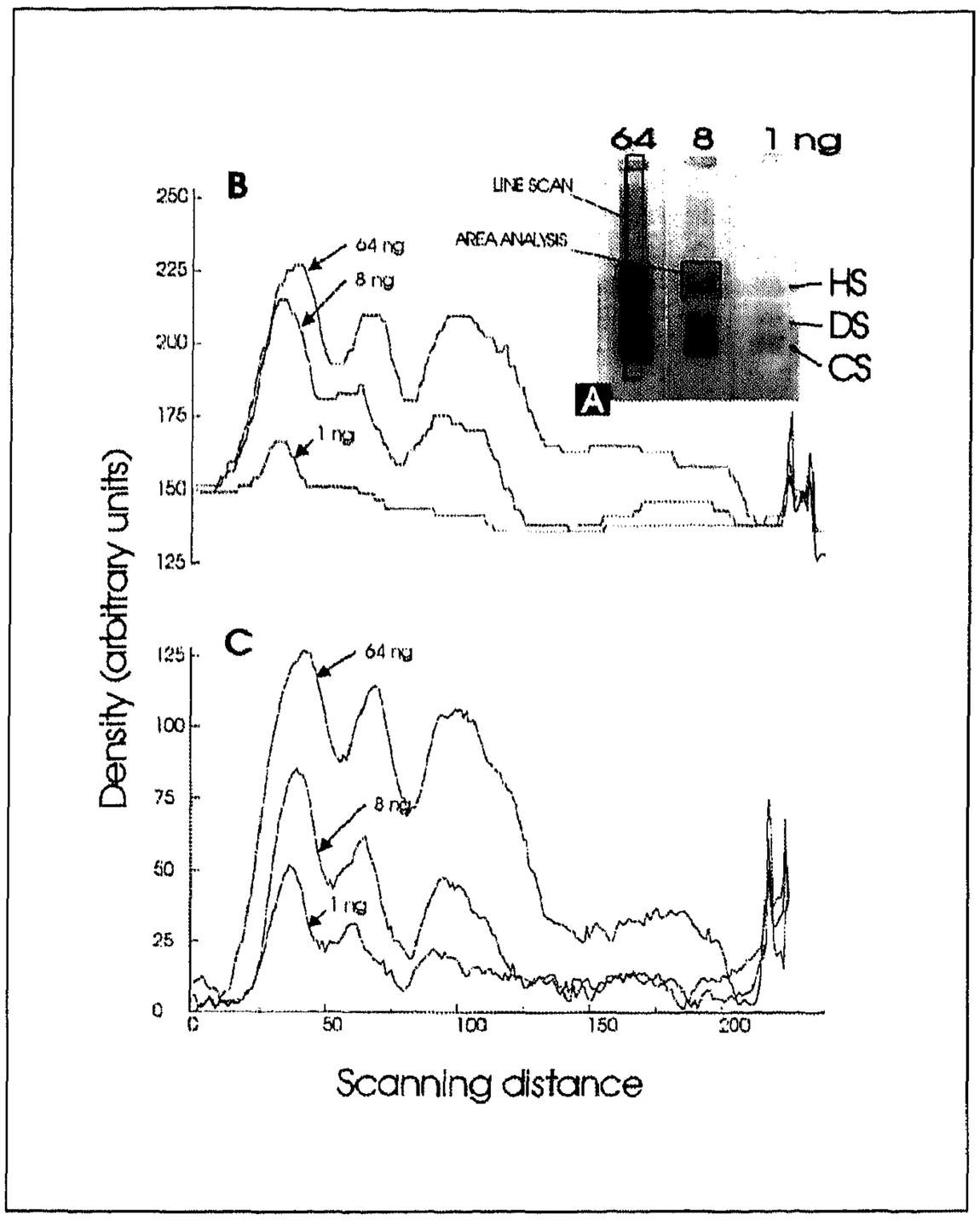

Figure 1. Comparison of densitometric analysis of an electropherogram of glycosaminoglycans (A), using an ordinary scanner plus ImageCalc (B) and a laser scanner plus additional software (C). A mixture of dermatan sulfate (DS), heparan sulfate (HS) and chondroitin sulfate (CS) was separated by agarose gel electrophoresis and stained with Azure A and silver (8). The marked areas in (A) visualize how band width and band area were selected.

Image Digitization

Gels or autoradiograms were digitized using an HP ScanJet™ II optical scanner (Hewlett-Packard, Corvallis, OR, USA). This scanner uses a CCD chip and normal white light for digitalization. The scanning parameters (i.e., white level, black level and contrast and brightness) were set in a way that a linear response was achieved over the maximum range of the scanned object. Briefly, the gel was pre-scanned (previewed) to view the "raw" image, and the area to be scanned was selected. Most scanning devices can display an intensity diagram of the selected area, which represents the number of pixels having a specific intensity. Contrast and brightness should be set in such a way that a linear response is obtained (usually this is the default setting). The black and white level should be set in such a way that all pixel values of the scanned area are within the interval 0 <pixel value <255. If the scanning device cannot display an intensity diagram, scanning parameters should be empirically determined. To evaluate if the settings are correct, the pixel values (which can be determined by ImageCalc) of the background and the most dense parts of the scan should be between 0 and 255 (values of about 30 for background and 220 for the densest parts ensure a linear response). It should be noted here that the error reading of most scanning devices that generate 8-bit images (i.e., desktop optical scanners that are not especially designed for quantitative analysis) is about 1 bit. The true dynamic resolution of such devices is therefore only 7-bit (128 gray levels), which poses some limitations to densitometric analysis.

Microscopic (fluorescence) images were digitized using the MagiCal hardware, which contains a sensitive 8-bit CCD camera connected to a fluorescence microscope. Image digitization, averaging and excitation filter settings were

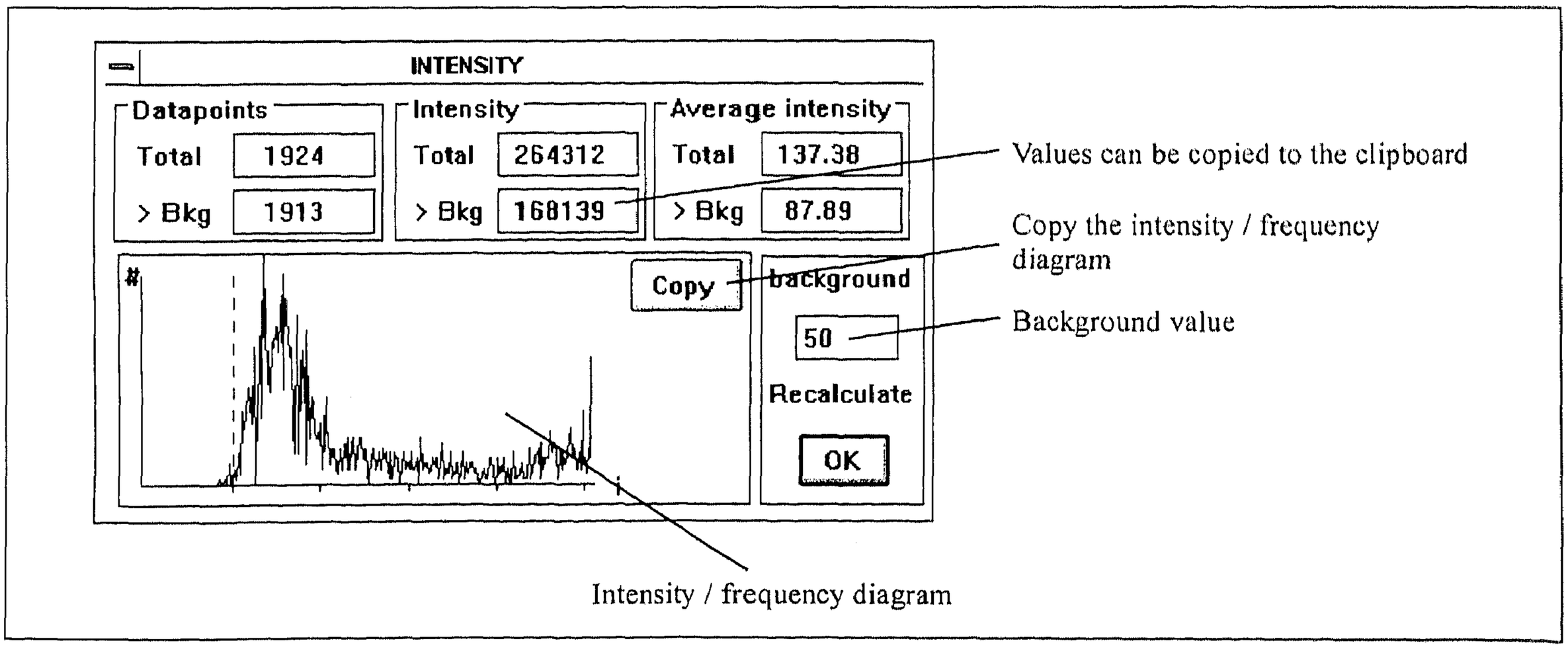

Figure 2. Dialog window of an analyzed image area, e.g., a band on an Azure A silver-stained gel. All values can be highlighted with the mouse and copied to the clipboard by pressing <Ctrl-C>, from where they can be retrieved into another MS-Windows application. The intensity (*i*)/frequency (#) diagram represents the pixel intensity frequency of the selected area. This can be applied for determination of the background level (dashed line) or for determination of the most common pixel values in images with irregular patterns.

performed by TARDIS software (developed solely for the MagiCal hardware) (Joyce-Loebl, Gateshead, UK). Images were stored by the TARDIS software in a 256 × 256 bitmap (256 gray levels) and imported into ImageCalc for further analysis. For more information on the TARDIS software and MagiCal hardware, see Reference 6.

Densitometric Analysis

Densitometric analysis of an image (e.g., a silver-stained gel) was performed in two ways: *(i)* by densitometric line scanning of the lanes of interest and *(ii)* by analysis of the total intensity of an area occupied by a stained band.

Densitometric Line Scanning Analysis Using ImageCalc

A selected area of the image was line-scanned in a vertical direction (i.e., from top to bottom) through the center of the lanes (see Figure 1A). The scan was displayed as the average pixel intensity of each horizontal line of the selected area. The scan data was copied to the Microsoft Windows "clipboard" from where it was retrieved into other window applications, i.e., Excel™ (Microsoft), for further processing. This scanning technique was compared with line scans performed by a laser scanner (UltroScan™ XL laser densitometer; Pharmacia Biotech, Uppsala, Sweden).

Densitometric Area Analysis Using ImageCalc

Areas of stained bands were selected by a rectangle fully surrounding the stained band (Figure 1A). Background was determined by selection of one or more areas in the vicinity of and of similar size as the band of interest. The total intensity of the stained area was calculated using ImageCalc, and the average pixel-background value was subtracted.

Separation and Detection of Glycosaminoglycans and [^{32}P]phosphatidic Acid

To examine if ImageCalc could be used for the quantitative analysis of silver-stained gels, glycosaminoglycans were separated by agarose gel electrophoresis using 0.05 M barium acetate as buffer (pH 5.0) and visualized by Azure A silver staining as described previously (8).

For analysis of autoradiograms using ImageCalc, a crude mixture of lipids containing [^{32}P]phosphatidic acid was separated on silica gel thin-layer chromatography (TLC) plates using a mixture of chloroform/acetone/acetic acid/methanol/H_2O (5/2/1/1.5/5, by vol). [^{32}P]Phosphatidic acid was detected by autoradiography, and the bands on the TLC plate were identified using the autoradiogram. The amount of ^{32}P was determined by scratching the phosphatidic acid from the TLC plate and counting the activity using a liquid scintillation counter. The size of the band areas for densitometry and ^{32}P measurement were equal in size. For background determination, a blank area of the TLC plate/autoradiogram was taken with similar dimensions as the band. For a critical evaluation of the settings for analysis of gels and autoradiograms, see Reference 5.

Determination of Cytosolic [Ca^{2+}] Concentration

Cytosolic [Ca^{2+}] in cultured human skeletal muscle cells was determined using the Fura-2 ratio technique (1,2). Fura-2 is a fluorescent Ca^{2+}-specific probe. Fura-2 loaded cells were excited at 340 and 380 nm. The ratio of the fluorescence at both wavelengths is linearly correlated with the cytosolic [Ca^{2+}] and is independent of the amount of Fura-2 present in the cell (1,2,6). Muscle cells were stimulated with 300 μM acetylcholine.

RESULTS AND DISCUSSION

Densitometric Line Scanning Analysis Using ImageCalc

To compare scanning images using a digitized gel plus ImageCalc with direct scanning using a laser scanner, glycosaminoglycans separated by agarose gel electrophoresis and detected by Azure A silver staining were analyzed (Figure 1). The laser scanner is not able to detect the minor bands (Figure 1B). In contrast, scanning images using white light plus ImageCalc detects the weakly stained bands well (Figure 1C). However, note that broad spectrum sources, i.e., white light, may give incorrect scanning results if a gel that has been stained by a specific dye like Coomassie blue is analyzed. In such cases a small spectrum light source of the complementary color gives best results. For CCD scanners, a colored transparent filter may be suitable.

The densitometric line scanning gives only a poor correlation between the density and the amount of glycosaminoglycans applied to the gel. We therefore analyzed gels/autoradiograms using densitometric area analysis.

Densitometric Area Analysis Using ImageCalc

Figure 2 displays the "measure" dialog window (as displayed by ImageCalc) of a selected image area. The data displayed in this dialog window is representative for the density of a selected area and can be copied to other window applications. The background of the selected area can be directly subtracted by entering the average background level. It can also be subtracted afterwards by selecting a part of the image

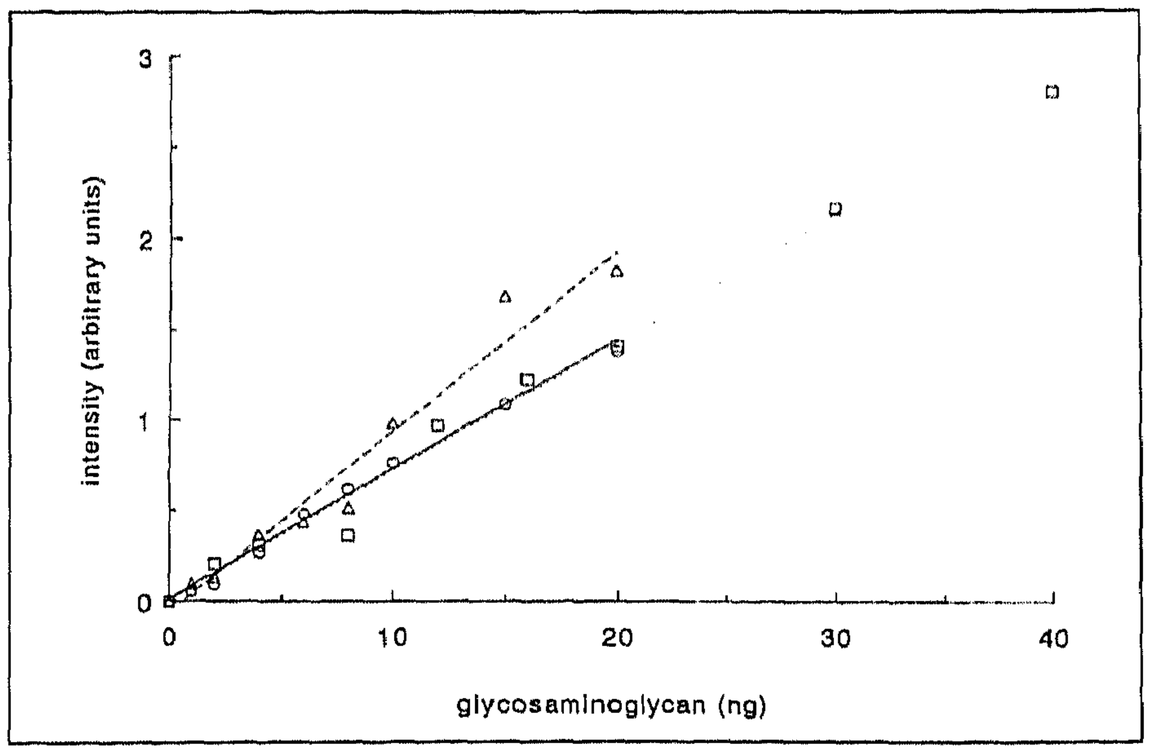

Figure 3. Densitometric analysis using ImageCalc. Various amounts of three types of glycosaminoglycans were separated by agarose gel electrophoresis and stained with Azure A and silver. The intensity of staining was determined using ImageCalc. Staining is linear from 1 to 20 ng for chondroitin sulfate (Δ) and dermatan sulfate (O) and from 1 to 40 ng for heparan sulfate (□). However, measurement accuracy diminishes with increasing range and, therefore, should be carefully assessed with standards in replicate experiments.

that is representative for the background. Figure 3 represents the results of the densitometric area analysis performed on three different types of glycosaminoglycans separated by agarose gel electrophoresis and stained with Azure A silver (8). The silver-stain density, as determined by digitalization with an optical scanner and densitometric analysis with ImageCalc, shows a very high correlation ($r = 0.98$) to the amount of glycosaminoglycan applied to the gel. Linearity is obtained for a range of 1 to 40 ng glycosaminoglycan.

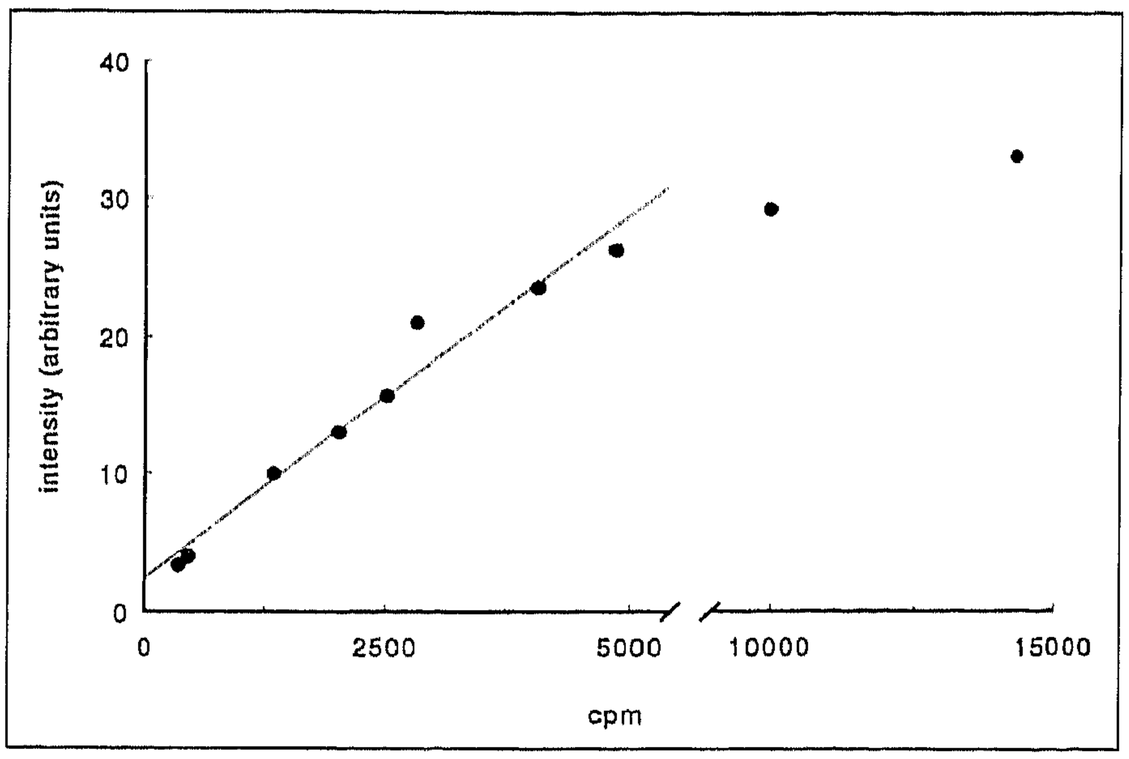

Figure 4. Densitometric analysis using ImageCalc. Crude mixtures of lipids containing [^{32}P]phosphatidic acid were separated by TLC, and the autoradiogram was analyzed using ImageCalc. The density of the [^{32}P]phosphatidic acid bands on the autoradiogram is linearly related to its amount for a range of 200 to 4000 cpm ($r = 0.94$).

Another example of the use of densitometric area analysis using ImageCalc is analysis of exposed films, e.g., autoradiograms. To illustrate this, [^{32}P]phosphatidic acid was separated by TLC and the amount of ^{32}P present in the phosphatidic acid spots of the TLC plate was compared with the intensity of the bands on the autoradiogram (Figure 4). The two parameters displayed a linear correlation ($r = 0.94$) over the range of 200 to 4000 cpm. However, note that photographic material (e.g., autoradiograms and photographs of gels) has a limited range of linearity (3,7). For quantitative use of photographic material, the range of linearity should be determined using proper standards.

Comparison and Analysis of Fluorescent Microscope Images

Besides densitometric analysis, ImageCalc can perform arithmetic calculations (i.e., add, subtract, divide, multiply and average) with the pixel values of a single image or of two related images (images having the same width and height parameters). An application in which this can be used is the analysis of related microscopic images, where the difference

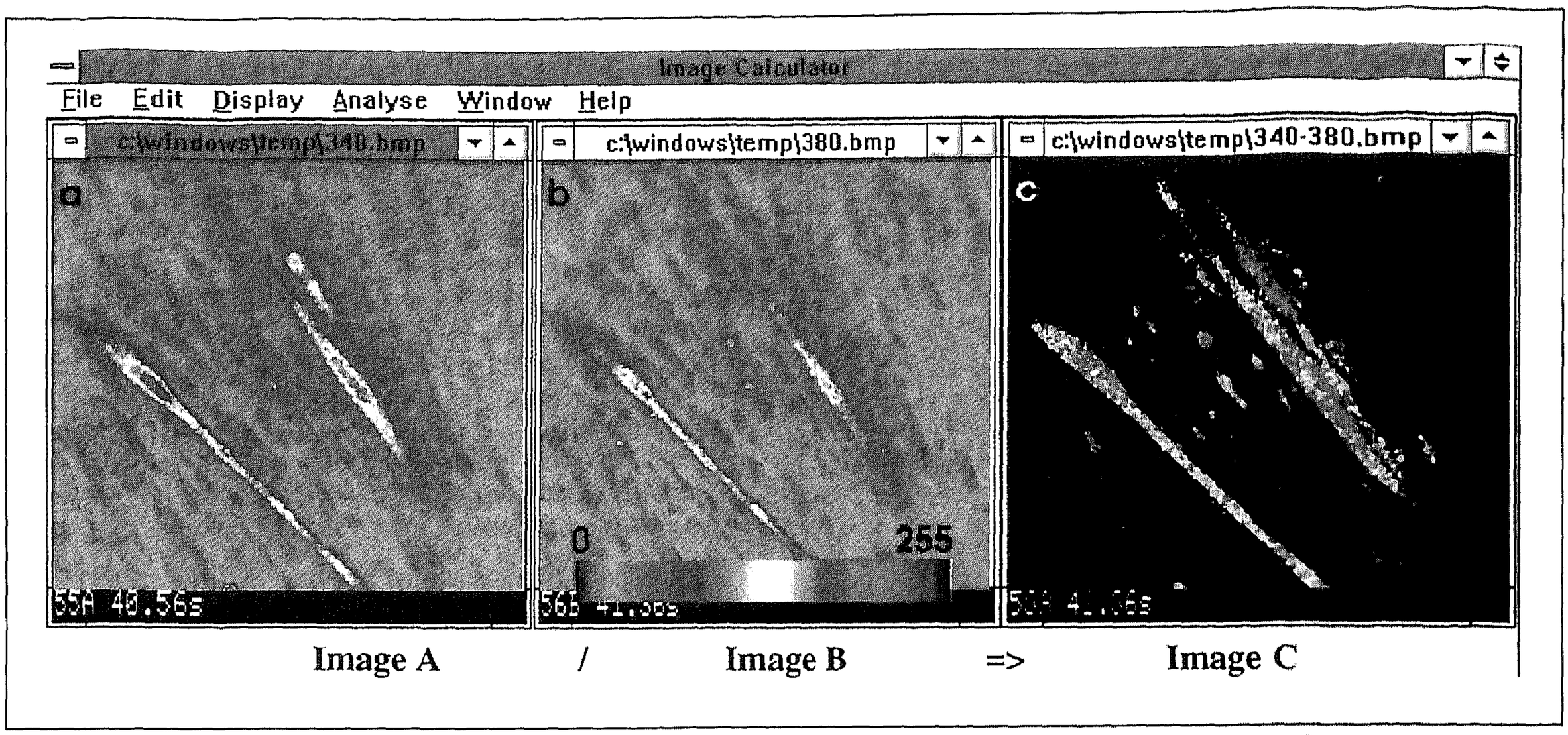

Figure 5. Use of ImageCalc in the analysis of cytosolic [Ca^{2+}] in cultured human skeletal muscle cells. Fura-2 was used as a Ca^{2+} probe and cells were stimulated with acetylcholine. Fura-2 loaded cells were exited at 340 nm (A) and at 380 nm (B), and the ratio of the fluorescence at each wavelength was determined using ImageCalc (C). The ratio represents the cytosolic [Ca^{2+}]. To maintain maximum range, the ratio values were expanded to values between 0 and 255, and all background values (values ≤30) were rejected from the calculation. For the determination of the background value see text.

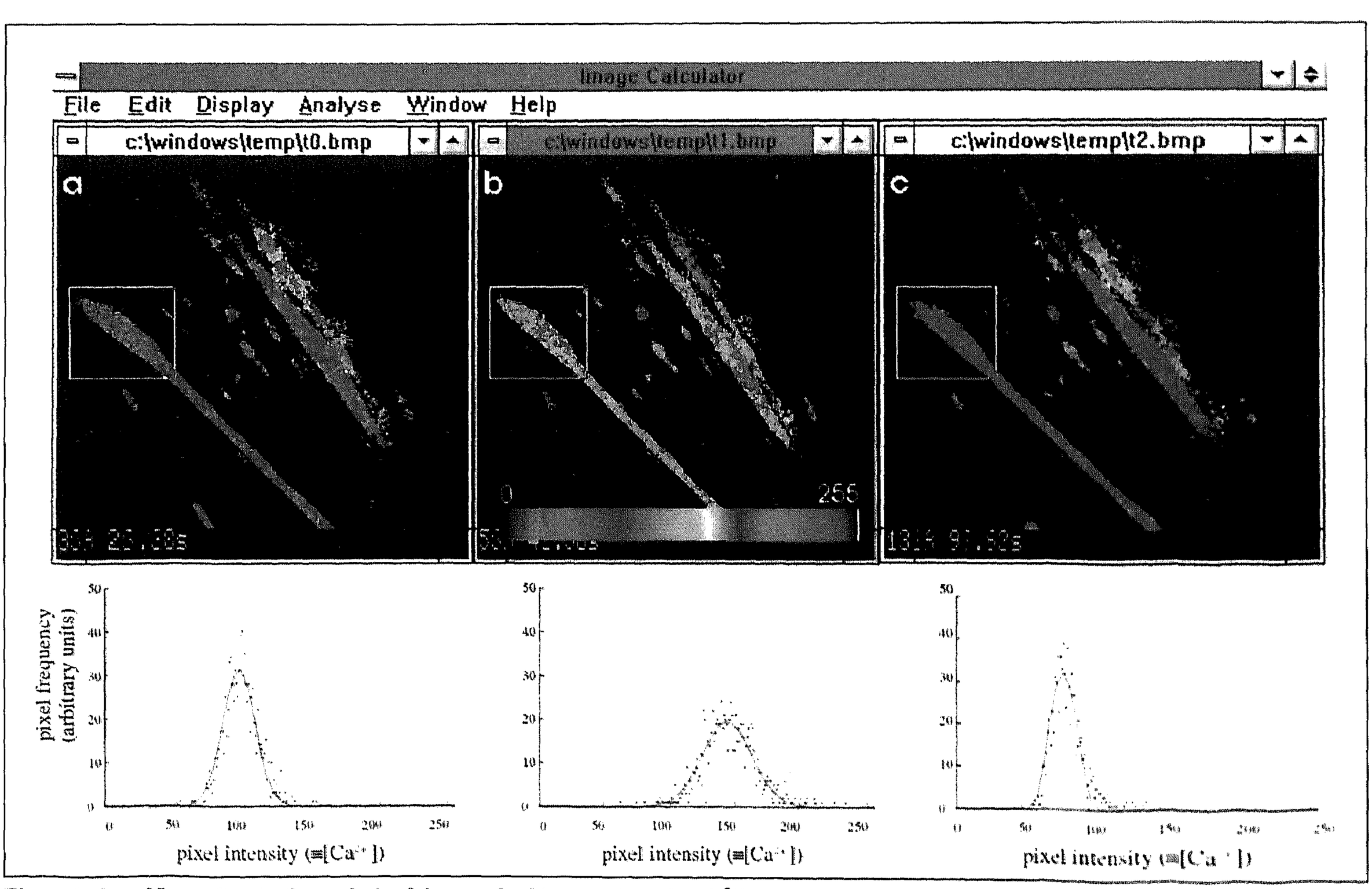

Figure 6. Use of ImageCalc in the analysis of time-resolved images. Cytosolic [Ca^{2+}] was determined in cultured human skeletal muscle cells using Fura-2 and the ratio technique. Cells were analyzed before (A), during (B) and after (C) stimulation with acetylcholine. The graphs underneath each image represent the pixel intensity frequency of the selected part of the image.

between the two related images is only due to a controlled change in one single parameter, e.g., time, excitation or emission wavelengths. As an example, we present the determination of the cytosolic [Ca^{2+}] in cultured human skeletal muscle cells using the Fura-2 ratio technique. ImageCalc was used to divide the image obtained at 340 nm by the image obtained at 380 nm (Figure 5), resulting in an image reflecting the cytosolic [Ca^{2+}] in the muscle cell. Since only a value of 0–255 can be attributed to a pixel, division (or multiplication) of two images may result in loss of data. ImageCalc can minimize this loss by expansion of the pixel value to the full 0–255 range. Furthermore, since division of two background values (i.e., noise) can result in high values, and thus erroneous analysis of [Ca^{2+}], a background cut-off value can be entered that results in rejection of all values below this cut-off value. The background cut-off value (30 in this experiment) was determined by measurement of the maximum value attributable to the background. This value can be read from the intensity/frequency diagram of a selected background area. However, it can also be selected empirically. Using ImageCalc, we analyzed the change in cytosolic [Ca^{2+}] as a result of acetylcholine stimulation (Figure 6). ImageCalc can also select corresponding areas of different images and, e.g., calculate the [Ca^{2+}] of a cultured human skeletal muscle cell at different time points (Figure 6).

In conclusion, ImageCalc is an affordable, Microsoft Windows-based computer program that allows easy analysis of digitized images. It can be used for the analysis of gels and autoradiograms as well as for computation and comparison of standalone and related (microscopic) images. Since all images stored in an uncompressed 8-bit format can be imported, ImageCalc is broadly applicable.

AVAILABILITY

ImageCalc can be obtained by sending a blank, MS-DOS® formatted diskette (either 5.25" or 3.5" high- or double-density) with a self-addressed envelope to Dr. T.H. van Kuppevelt, Department of Biochemistry, University of Nijmegen, P.O. Box 9101, 6500 HB Nijmegen, The Netherlands. The source code is available upon request. The program is released as "shareware" and may be used for noncommercial purposes only. If you find the program useful, we ask you to support the "Dutch Asthma Foundation" with a contribution of at least $25.

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